Journal of Chromatography A, 1209 (2008) 1–9

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development of an immunoaffinity column method using broad-specificity monoclonal antibodies for simultaneous extraction and cleanup of quinolone and sulfonamide antibiotics in animal muscle tissues

Cun Li[a,b,c,1], Zhanhui Wang[a,b,1], Xingyuan Cao[a,b], Ross C. Beier[d], Suxia Zhang[a,b], Shuangyang Ding[a,b], Xiaowei Li[a,b], Jianzhong Shen[a,b,*]

[a] Department of Pharmacology and Toxicology, College of Veterinary Medicine, China Agricultural University, Beijing 100094, China
[b] National Reference Laboratories for Veterinary Drug Residue, District Haidian, Beijing 100094, China
[c] Department of Veterinary Medicine, College of Agriculture, Hebei University of Engineering, Handan 056038, China
[d] Southern Plains Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, 2881 F & B Road, College Station, TX 77845-4988, USA

ARTICLE INFO

Article history:
Received 7 July 2008
Received in revised form 13 August 2008
Accepted 18 August 2008
Available online 6 September 2008

Keywords:
Immunoaffinity
Quinolones
Sulfonamides
Muscle tissues
Residue analysis
LC–ESI-MS/MS

ABSTRACT

This paper describes a novel mixed-bed immunoaffinity column (IAC) method. The IAC was produced by coupling anti-quinolone and anti-sulfonamide broad-specificity monoclonal antibodies to Sepharose 4B for simultaneously isolating 13 quinolones (QNs) and 6 sulfonamides (SAs) from swine and chicken muscle tissues, followed by antibiotic determination using liquid chromatography–tandem mass spectrometry (LC–MS/MS). A new broad-specificity Mab (B1A4E8) toward sulfonamides was produced using sulfamethoxazole as hapten that demonstrated cross-reactivities to 6 SAs in the range of 31–112%. IAC optimized conditions were found that allowed the IAC to be reused for selective binding of both SAs and QNs. Recoveries of all 19 antibiotics from animal muscle ranged from 72.6 to 107.6%, with RSDs below 11.3% and 15.4% for intra-day and inter-day experiments, respectively. The limit of quantification ranged from 0.5 to 3.0 ng/g. The strategy used here for a mixed-bed IAC may be used to study other compounds and more than two classes of analytes simultaneously.

1. Introduction

A variety of antibiotics are used in stockbreeding or aquiculture for treating infections, as well as for growth-promotion. As a result, these antibiotics can be present in food products of animal-origin and may pose a health threat to consumers [1]. To prevent potential health problems for consumers, some countries like the European Union and the United States have established maximum residue limits (MRLs); for example: quinolones (QNs) and sulfonamides (SAs) [2,3].

Due to the complexity of samples, elaborate cleanup steps must be involved prior to quantification of target antibiotics. Sample preparation procedures frequently include several steps, such as solvent extraction, defatting with hexane, and solid phase extraction that have been widely employed in the last decade [4]. However, antibiotic retention is often based on hydrophobic interactions and the co-extraction of antibiotics and matrix interfering substances may occur during solid phase extraction.

Immunoaffinity column chromatography (IAC), using analyte specific antibodies, is based upon molecular recognition and can provide an alternative method to isolate, purify, and concentrate target analytes from complex sample matrices [5]. The IAC extraction of a variety of analytes, such as bisphenol A [6], atrazine [5], and sufonamides [7] from complex matrices followed by detection with different devices have been reported. Most IACs have been specific for only one type of antibiotic, while others were developed to bind multiple-drugs, which belong to the same group or are structurally related compounds [7,8]. Furthermore until recently, all IACs were prepared using a single preparation of polyclonal or monoclonal antibody that had either narrow-specificity or broad-specificity immobilized on a solid support. However, Martin-Esteban et al. [8] recently reported the immunoaffinity-based extraction of phenylurea herbicides using a cocktail or mixture of antibodies produced against isoproturon and chlortoluron in order to extract the whole group of compounds. In addition, Pichon et al. [9] discussed in a review the great potential of a mixed-bed anti-phenylurea/triazine immunosorbent cartridge for multi-residue determination of

* Corresponding author at: China Agricultural University, Beijing 100094, China. Tel.: +86 10 6273 2803; fax: +86 10 6273 1032.
E-mail addresses: sjz@cau.edu.cn, hhlicun@live.cn, licun2006@yahoo.cn (J. Shen).
[1] Cun Li and Zhanhui Wang contributed equally to this work.

0021-9673/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2008.08.116

several phenylurea and triazine herbicides from surface water, and Chan et al. [10] and Trucksess et al. [11] described the simultaneous determination of aflatoxins and ochratoxin A using an automated commercial IAC followed by liquid chromatography with fluorescence detection. There is limited information concerning an IAC that simultaneously extracts different groups of antibiotics, and these previous IAC procedures have used polyclonal antibodies rather than Mabs [7,12]. The interest for extracting, concentrating and cleanup of different classes of antibiotics from complex matrices is increasing [9]. This strategy can be implemented by preparing a single IAC packed with different antibodies specific to different antibiotics, and then optimizing the loading and desorption conditions to simultaneously elute the target antibiotics. This will increase sample throughput and reduce the cost of consumables.

QNs and SAs represent classes of synthetic antibiotics that are widely used in veterinary medicine [13,14]; therefore, these two classes of antibiotics were selected as model analytes to evaluate the possible utilization of a single IAC to extract, concentrate, and cleanup these two classes of structurally unrelated antibiotics. In the field of QN and SA residue analysis, only a few applications of IAC procedures have been reported. Märtlbauer et al. [15] described a monoclonal antibody (Mab)-based IAC for the detection of sulfamethazine (SMZ) and sulfadiazine (SDZ) in milk. Heering et al. [16], Crabbe et al. [12], and Li et al. [7] reported polyclonal antibody-based IACs for the determination of sulfathiazole (STZ) in honey [16], SMZ and its major metabolites in urinary samples [12], and multiple-SAs in swine meat using a group-selective antibody against the common structure of SAs [7]. For the application of IACs to QN cleanup, only Holtzapple et al. [17] have described several procedures for the extraction of not more than four QNs using one Mab produced against sarafloxacin [12,18,19]. Although there are recent reports of the production of broad-specific polyclonal antibodies for SAs [3,18] and QNs [20], IAC preparation using these antisera have not yet been described. One reason for this may be that using a polyclonal antibody does not guarantee a long-term supply of antibody, and further production of polyclonal antibody would require new animals and the resultant antisera would not be equivalent.

In this paper we describe a novel IAC prepared from a previously obtained [19] group-specific Mab (C4A9H1) produced against ciprofloxacin, which recognized 12 QN analogs with uniform cross-reactivity of 35–100% and a new broad-specificity Mab (B1A4E8) produced against sulfamethoxazole (SMX) that recognizes 6 SA analogs. To the author's knowledge, this is the first report of a broad-specificity Mab to SAs that has been used for IAC, and also used in combination with a broad-specificity anti-QN Mab for simultaneous determination of both QNs and SAs by IAC. This study focuses on the generation and application of a single IAC using a mixture of two broad-specificity Mabs covalently immobilized on Sepharose used to simultaneous determine two different groups of antibiotics (19 antibiotics total) with further antibiotic quantification by liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS). Binding capacity of the IAC, effect of flow rate on recovery, and the eluate composition were studied. The novel strategy was evaluated as an effective analytical method for the simultaneous detection of QNs and SAs in swine and chicken muscle.

2. Experimental

2.1. Reagents and chemicals

The QN Mab (C4A9H1) and the chloramphenicol (CAP) Mab (used as a negative IAC control) were previously produced against ciprofloxacin (CIP) and CAP, respectively, by our group [19,21]. Enrofloxacin (ENRO) (purity: 100%), CIP hydrochloride (100%), norfloxacin (NOR) (99.6%), flumequine (FLU) (99.5%), pefloxacin methanesulfonate (PEF) (99.9%), sarafloxacin (SAR) (99.6%), and difloxacin (DIF) (98.4%) were purchased from the China Institute of Veterinary Drug Control (Beijing, China). Ofloxacin (OFL) (≥99.0%), lomefloxacin (LOM) (99.8%), enoxacin (ENO) (>99.0%), danofloxacin (DANO) (≥99%), oxolinic acid (OXO) (≥99%), marbofloxacin (MARB) (≥99%), SMX (99.9%), SDZ (99.9%), sulfapyridine (SPY) (99.0%), STZ (99.9%), sulfamethizole (SMT) (99.0%), sulfamonomethoxine (SMM) (99.0%), bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The myeloma cell line SP2/0 was a gift from Professor Jixun Zhao (China Agricultural University, Beijing, China). Dulbecco's Modified Eagle Media (DMEM) used for cell culture was obtained from Huamei (Beijing, China). CNBr-activated Sephrose 4B (46–165 μm) was purchased from Pharmacia Corporation (Uppsala, Sweden). HPLC grade acetonitrile (ACN), methanol (MeOH) and formic acid were obtained from Fisher Scientific Inc. (Pittsburgh, PA, USA). All other chemicals and solvents were of analytical grade or better and were obtained from Beijing Chemical Reagent Co. (Beijing, China). Deionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Standard solutions and buffers

Individual stock standard solution of QNs (100 μg/mL) was prepared by dissolving 5.00 mg of each QN standard with 2 mL 0.03% NaOH and diluting to a final volume of 50 mL with MeOH. Individual stock standard solutions of SAs (100 μg/mL) were prepared by dissolving 5.00 mg of each SA standard and diluting to a final volume of 50 mL with MeOH. Mixed fortifying standard solutions (1 μg/mL for OXO and FLU, 2 μg/mL for OFL, PEF, NOR, SPY, LOM, DANO, ENRO, STZ, DIF, SMM and SMX, 4 μg/mL for ENO, SDZ, CIP and SMT, 6 μg/mL for MARB and SAR) were prepared by diluting and mixing each appropriate standard solution and diluting to a final volume of 100 mL with MeOH.

The buffers that were used in the ELISA format are found in Wang et al. [19]. Phosphate buffered saline (PBS) was prepared by dissolving 0.2 g KH_2PO_4, 0.2 g KCl, 2.9 g $Na_2HPO_4{\cdot}12H_2O$, and 8.8 g NaCl in 1 L of water, and the pH was adjusted to 7.4 with 1 M NaOH. The HCl solution (0.001 M) was prepared by dissolving 84 μL HCl (37%) in 1 L of water. The $NaHCO_3$ solution (0.1 M, pH 8.4) was prepared by dissolving 8.4 g $NaHCO_3$ and 29.3 g NaCl in 1 L of water. Tris–HCl buffer (0.1 M, pH 8.0) was prepared by dissolving 12.1 g Tris (tris(hydroxymethyl)aminomethane), 29.3 g NaCl, and 2.4 mL HCl (37%) in 1 L of water. Acetate buffer (0.1 M, pH 4.0) was prepared by dissolving 2.5 g $CH_3COONa{\cdot}3H_2O$, 29.3 g NaCl, and 4.7 mL glacial acetic acid in 1 L of water. PBS containing 0.01% (v/w) sodium azide solution was prepared by dissolving 0.2 g NaN_3 in 1 L of PBS.

2.3. Instrumentation

Polystyrene microtiter plates were purchased from Beijing Wanger Bio-technology Co., Ltd. (Beijing, China). The ELISA plate reader was obtained from Tecan Inc. (Tecan Sunrise, Durham, NC, USA). The ultraviolet–visible (UV–Vis) spectrometer was obtained from Shanghai Analytical Instrument (type 751GW, Shanghai, China). The vortex mixer was from Fischer Scientific (Norcross, GA, USA) and the centrifuge was purchased from Hettich GmbH and Company oHG (model Mikro 22 R, Kirchlengern, Germany).

The LC equipment was a Waters Alliance 2690 quarternary solvent delivery system (Waters, Milford, MA, USA). The chromatographic separation of the QNs and SAs was performed using a Waters Symmetry Shield RP18 (150 mm × 2.1 mm i.d., 3 μm) column. Mobile phase consisted of 0.1% formic acid aqueous solution

Table 1
LC mobile phase gradient program[a]

Minutes	A (%)	B (%)	C (%)	Curve
0	95	5	0	1
5	85	5	10	6
10	70	20	10	6
15	10	80	10	6
16	10	90	0	6
17	95	5	0	1
30	95	5	0	1

[a] Solvent A, water with 0.1% formic acid; solvent B, ACN with 0.1% formic acid; solvent C, MeOH.

(solvent A), 0.1% formic acid ACN solution (solvent B), MeOH (solvent C), and the gradient is shown in Table 1. The flow rate was set to 0.2 mL/min and the run time was 35 min for each sample. Injection volumes were 20 μL and all separations were controlled at 20 °C.

The ESI-MS/MS detection of the QNs and SAs was achieved using a Quattro LC triple stage quadrupole instrument from Micromass (Manchester, UK). Positive ions were acquired in the multiple reaction monitoring (MRM) mode using a desolvation temperature of 300 °C and a source temperature of 80 °C. Nitrogen was used as the nebulization and desolvation gas, at flow rates of 28 and 450 L/h, respectively. Argon was used as the collision gas. Cone voltage and collision energy were optimized for each analyte separately. The two most abundant product ions were monitored using the conditions given in Table 2, but only one ion was used for quantification (indicated in Table 2).

2.4. Synthesis of immunogen and coating antigen

SMX was bound to BSA using glutaraldehyde as the coupling reagent according to Märtlbauer et al. [22]. Briefly, SMX (350 mg) was mixed with BSA (600 mg) in 75 mL of PBS (0.01 M, pH 7.3)/dioxane (2:1, v/v). Glutaraldehyde (0.35 mL, 25%) was added drop-wise to the mixture, which was stirred at room temperature for 3 h. The reaction mixture was dialyzed for 3 days against three changes of PBS.

The coating antigen consisting of SMX-OVA was synthesized by a diazo-method [23]. SMX (150 mg) was dissolved in 12 mL of 0.5N sulfuric acid and kept at 4 °C, and then sodium nitrite (57 mg) in 3 mL distilled water was added to the SMX solution and cooled in chopped ice for 15 min. The final SMX solution was then added drop-wise with stirring to the OVA (300 mg) in 8 mL sodium carbonate. The pH of the reaction mixture was kept at 10 by addition of sodium hydroxide (1 M). The reaction mixture was stirred for 6 h at room temperature and then dialyzed against 0.9% sodium chloride for 3 days (one change every 8 h).

2.5. Production of monoclonal antibodies

The procedures used for generating the immune response in mice and producing Mabs were similar to those described by Zhang et al. [23].

2.6. ELISA methods and molecular modeling

The ELISA protocols used for SAs were similar to that used for QNs previously described by Wang et al. [19]. A four parameter logistic equation was used to fit the immunoassay data. Calculations were performed using OriginPro 7.5 software (Origin Lab Corporation, Northampton, MA). The IC_{50} value represents the analyte concentrations obtained at 50% inhibition. Cross-reactivity (CR) was calculated according to the equation,

$$CR = \frac{IC_{50}\,(SMX)}{IC_{50}\,(Sulfanamide)} \times 100\%$$

where IC_{50} is the concentration at which 50% of the Mab is bound to the analyte and has the units of pmol/mL.

2.7. Immunoaffinity column preparation

The immunosorbent was produced according to the manufacturer's instructions and related literature [7,24]. One g of CNBr-activated Sepharose 4B (1 g of powder results in about 3.5 mL final volume of gel) was dissolved in 5 mL of HCl (0.001 M) and poured into a sintered-glass funnel (40–60 μm). The gel was washed with 200 mL of HCl (0.001 M), and then with 600 mL of $NaHCO_3$ solution (0.1 M, pH 8.4). After this procedure, the gel was mixed with a total of 28 mg Mab (8 mg/mL gel), 14 mg anti-CIP Mab (C4A9H1) and 14 mg anti-SMX Mab (B1A4E), dissolved in 5 mL $NaHCO_3$ (0.1 M, pH 8.4), and gently stirred at 4 °C for 20 h. The mixture was washed with 50 mL PBS to remove the un-reacted Mabs. The eluted solution was collected to detect the amount of Mabs by UV–Vis spectrometry and the coupling efficiency was determined. The mixture was redissolved in 10 mL Tris–HCl buffer (0.1 M, pH

Table 2
MS/MS method parameters

Analytes	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Dwell time (s)	Cone voltage (V)	Collision energy (eV)	Retention time window (min)
MARB	9.53	363	344.8[a]/319.8	0.3/0.3	30/30	20/15	6.0–11.0
ENO	10.68	321	303[a]/239.4	0.1/0.1	35/35	20/20	6.1–12.0
OFL	11.12	362.1	318.1[a]/261	0.1/0.1	35/35	20/25	6.5–12.0
SDZ	11.07	251	155.9[a]/107	0.2/0.2	22/22	15/20	6.6–12.0
PEF	11.68	334	316[a]/290	0.2/0.2	35/35	20/20	7.0–14.0
NOR	11.93	320	302/276[a]	0.2/0.1	35/35	20/20	7.2–14.0
SPY	12.12	250	155.9[a]/107.9	0.1/0.1	25/25	20/20	7.4–14.0
CIP	12.19	332	287.8[a]/244.74	0.1/0.1	35/35	20/20	7.8–15.0
LOM	12.26	352	308/265[a]	0.2/0.2	35/35	20/20	8.0–15.0
DANO	12.54	358.1	340[a]/314	0.2/0.2	35/35	20/20	8.2–15.0
ENRO	12.70	360.1	316.2[a]/245	0.1/0.1	35/35	20/25	8.4–15.0
STZ	12.92	256	155.9[a]/107.9	0.1/0.1	22/22	18/18	8.6–16.0
DIF	13.43	400	356[a]/299	0.1/0.1	35/35	20/25	10.0–17.0
SAR	13.56	386	368/342[a]	0.1/0.1	40/40	20/25	10.5–17.0
SMT	16.05	271	155.8[a]/107.8	0.3/0.3	30/30	15/20	12.0–20.0
SMM	17.05	281	155.9[a]/125.8	0.1/0.1	22/22	18/18	17.0–23.0
SMX	17.43	254	155.9[a]/107.8	0.05/0.05	22/22	18/18	18.0–24.0
OXO	17.84	262	244[a]/215.9	0.1/0.1	30/30	20/25	20.0–30.0
FLU	18.80	262	243.9[a]/201.9	0.1/0.1	30/30	20/30	20.0–30.0

[a] Ion used for quantification.

8.0) to block the un-reacted sites on the CNBr-activated Sepharose 4B at 4 °C for 2 h, and the gel was washed alternately with 3 cycles of 20 mL acetate buffer (0.1 M, pH 4.0) and 20 mL Tris–HCl buffer. Finally, 1 mL bed volume gel was transferred to a glass column (10 mm × 0.8 mm i.d.), and stored in PBS containing 0.01% (v/w) sodium azide at 4 °C. The same procedure was used to obtain a control Sepharose column without the specific Mab and a control column containing gel with 8 mg/mL of the anti-CAP Mab [21].

2.8. Column capacity determination

A relatively large amount (6000 ng) of each QN and each SA (60 μL of stock standard solution) was mixed with 40 mL of PBS containing 15% MeOH. These solutions were drawn through the IAC (pre-conditioned with 10 mL of PBS) at 1 mL/min. The antibiotic-saturated column was washed with 20 mL of PBS and 30 mL of water. Finally, 4 mL of MeOH/water/ammonia (90:9.8:0.2, v/v/v) was used to elute the analytes. The eluate was evaporated to dryness by a stream of N_2 at 45 °C. The residue was reconstituted in 0.5 mL of 0.1% formic acid solution. After filtering the reconstituted samples through a 0.2 μm PTFE filter (Jinteng Ltd., Tianjin, China), they were injected into the LC–MS/MS system. The column was regenerated by equilibrating with 10 mL of water and 20 mL of PBS, and stored in PBS–0.01% sodium azide (v/w) at 4 °C when not in use.

2.9. Sample preparation for IAC

Two grams (±0.01 g) of muscle tissue homogenate was weighted into a 50 mL polypropylene centrifuge tube. About 8 mL MeOH/water (8:2, v/v) was added to the sample and vortexed for 1 min, and then shaken for 30 min with an orbital shaker (model HY-4, Xinrui Automatic Apparatas Co., Shanghai, China). After centrifugation at 3000 × *g* for 10 min, the supernatant was decanted into a clean tube. The pellet was re-extracted with an additional 8 mL MeOH/water (8:2, v/v), vortexed, and centrifuged as before. The supernatants were combined and vortexed for about 10 s. Some of the combined supernatant (8 mL) was transferred and diluted with 35 mL PBS. This solution was subjected to IAC cleanup. The cleanup procedures (loading, washing, elution, and regeneration) were the same as described in the column capacity determination section.

3. Results and discussion

3.1. Monoclonal antibodies and molecular modeling study

The antibody is the key reagent in the IAC process. It determines the potential use of the immunosorbent, whether the IAC is used for a single compound or for class-selective purposes. Both polyclonal antibodies and Mabs have been selected as the target antibody for IAC, with an increase in the use of Mabs in recent years. Although Mab production is more costly and IAC using Mabs is more susceptible to organic solvents, which can cause a relatively rapid decrease in column capacity, it does guarantee a long-term availability of reproducible antibody without requiring animals for further large-scale production [25]. In previous studies, several IAC's have been demonstrated for extraction and cleanup of small analytes such as α-zearalenol and related compounds [26], *s*-triazines [27], and QNs [28] from complicated samples. In those cases, if the compounds of interest were present in the samples at high concentrations and were loaded onto the IAC while using antibodies of non-uniform affinity to all analogs in the group, the competition between the structurally related analogs for the limited antibody binding sites will occur, and potentially cause the desorption of an unrelated distribution of analytes in respect to the true sample distribution. The first important step in preparing a robust class-selective IAC was to produce the proper antibody that can trap structurally related analogs to the same extent and show an equal cross-reactivity with all analytes involved. In a previous paper, a Mab (C4A9H1) against CIP was produced that showed similar affinity to 12 different QNs [19]. According to the published data, the C4A9H1 Mab had a 100% cross-reactivity to CIP with a 35–82% cross-reactivity to 11 other QNs; but only a 3.1% and 2.9% cross-reactivity was obtained for DIF and SAR, respectively [19]. Due to the robust specificity and selectivity of the C4A9H1 Mab, it was utilized to prepare a class-selective IAC for extracting 13 QNs, not including amifloxacin (AMI).

Table 3
Column capacity for the analyte and specificity of antibody

Analyte	Class	CC[a]		IC_{50}		CR[b] (%)
		ng/mL gel	pmol/mL gel	ng/mL	pmol/mL	
MARB	QNs	1630	4490	21.5	59.2	45
ENO		2216	6903	20.0	62.3	43
OFL		2099	5798	16.5	45.6	58
PEF		2183	6536	18.0	53.9	50
NOR		2464	7700	12.5	39.1	69
CIP		2387	7190	8.9	26.8	100
LOM		1941	5514	22.0	62.5	43
DANO		1826	5101	16.5	46.1	58
ENRO		2064	5733	10.9	30.3	88
SAR		1552	4021	307.0	795.3	3.4
DIF		1402	3505	284.0	710.0	3.8
OXO		1711	6531	22.0	84.0	32
FLU		1893	7225	25.5	97.3	28
SDZ	SAs	2106	8390	3.6	14.3	58
SPY		2398	9592	6.8	27.2	31
STZ		1950	7617	1.9	7.4	112
SMT		1834	6768	3.4	12.5	66
SMM		2156	7673	3.6	12.8	65
SMX		1923	7571	2.1	8.3	100

[a] CC = column capacity.
[b] CR = cross-reactivity, and it was calculated using IC_{50} values with units of pmol/mL.

To simultaneously trap and extract both QNs and SAs from the sample matrix, a single column was used that contained a mixture of both anti-SA and anti-QN Mabs immobilized on gels. In this study a new Mab was produced against SMX, and the affinity or IC_{50} values to 6 SAs was determined by conventional ELISA procedures and the IC_{50}'s were shown in Table 3. The Mab (B1A4E8) showed cross-reactivity with 6 SAs in the range of 31–112% (Table 3).

The two broad-specificity Mabs (B1A4E8 and C4A9H1) with uniform affinity toward their corresponding analogs were used to prepare an IAC that could simultaneously trap both QNs and SAs in one single column.

3.2. Preparation of the immunoaffinity column

The aim of this study was to extract two groups of antibiotics (QNs and SAs) using a single IAC column; therefore, two different antibodies with different binding characteristics were immobilized on solid support material and the two derivatized gels were combined to form one IAC. In this case, CNBr-activated Sepharose 4B was employed because it is chemically and biologically inert, easily derivatized, and is appropriate for use in off-line coupling with separation techniques [25]. Some important veterinary drugs have been extracted and cleaned up using an IAC prepared with this gel in our lab [21,24,26,29]. Five- to ten-mg amounts of antibody per milliliter of gel is recommended [12]. An amount of 14 mg of the C4A9H1 Mab and 14 mg of the B1A4E Mab were immobilized

Table 4
Influence of the immobilized Mab on column capacity and coupling efficiency

Mabs	Column capacity (ng/mL)		Coupling efficiency (%)
	CIP	SMX	
C4A9H1	2316	0	91.0
B1A4E	0	1896	89.3
C4A9H1 + B1A4E	2379	1931	91.1
CAP	0	0	89.2

on 3.5 mL of CN-Br-activated Sepharose 4B. The total amount of 8 mg/mL gel using these two different Mabs is equivalent to the loading of only one type of Mab, which was frequently used in our previous studies [24,29]. In order to investigate whether the two Mabs influenced Mab immobilization, column capacity, and coupling efficiency, three immobilization procedures were evaluated as follows: (i) anti-CIP Mab (C4A9H1, 4 mg); (ii) anti-SMX (B1A4E, 4 mg); (iii) 4 mg each of the two Mabs were simultaneously immobilized on 1 mL of gel. The column capacities for CIP and SMX and coupling efficiency of the Mabs were separately or simultaneously measured according to the method described in the column capacity determination section. The result was summarized in Table 4. The column capacities for CIP and for SMX individually from the three preparation procedures resulted in close values, but the column capacity for CIP was significantly different from the column capacity for SMX ($P = 0.0068$). However, both of these capacities are of reasonable value to be used for IAC. After coupling the Mabs and blocking the remaining active groups, the coupling efficiency of three different column preparations using the above procedures was measured, and showed that the gel contained 3.64 mg/mL gel anti-CIP Mab, 3.57 mg/mL gel anti-SMX Mab, 7.23 mg/mL gel anti-CIP Mab and anti-SMX Mab, respectively, which corresponds to an average coupling efficiency of 91.1%. Due to a satisfactory column capacity, uniform coupling efficiency, less handling time, and cost-effectiveness, the procedure that simultaneously immobilized the two Mabs on the gel was employed. To investigate whether the analytes of interest were trapped by the IAC because of non-specific interactions, the two control columns containing 8 mg/mL gel of the anti-CAP Mab (89.2% coupling efficiency) and no Mab were used to evaluate the retainability of CIP and SMX. The capacity of the two control columns for CIP and SMX was approximately zero, indicating that the retention of CIP and SMX in IAC due to non-specific antibody interactions was non-existent (Table 4).

3.3. Evaluation of the IAC conditions

Evaluation of loading, washing, flow rate, and elution conditions is necessary when beginning IAC because these conditions may have a strong influence on the association and dissociation of antigen–antibody binding and therefore, analyte recovery. In the case of single antibody IAC, common buffers or organic solvents may be employed to extract the analytes by IAC, but in multi-antibody IAC the solution conditions must be carefully evaluated. In particular, the IAC developed here is different from previous ones because here there are two Mabs with different specificities immobilized on the same column. A difficulty with using a mixed-bed IAC for simultaneous extraction of QNs and SAs was that a proper compromise of conditions must be found for these two groups of analytes.

3.3.1. Loading conditions

To simplify the sample pretreatment procedure is the primary goal in developing methods for the determination of veterinary drugs in complicated samples. Unlike the analysis of environmental samples, like wastewater, during the analysis of food of animal-origin samples, like muscle tissue, the first extraction step often uses an organic solvent for adequate dissolution of the analyte of interest. QNs and SAs are soluble in polar organic solvents, and are usually extracted with MeOH, ACN, ethyl acetate, acetone, or mixtures of these organic solvents. In our experiments, the sample extract was directly loaded onto the IAC. In order to study whether the loading medium had an effect on analyte recovery, solutions containing small amounts of MeOH were used. An amount of 40 mL of CIP and SMX, selected as the model analytes, at a level of 20 ng/mL were loaded in PBS, PBS–MeOH (95:5, v/v), PBS–MeOH (90:10, v/v), or PBS–MeOH (80:20, v/v) and washed with 20 mL of PBS followed by 30 mL of water, and finally eluted with 4 mL of MeOH/water/ammonia (90:9.8:0.2, v/v/v). The column effluents were evaluated by LC–MS/MS. Recovery results demonstrated that adding MeOH in the loading medium up to 20% showed no significant influence on recovery. A decrease in recovery from 94 to 89% and 92 to 87% for CIP and SMX, respectively, was observed as the MeOH increased from 0 to 20%. Since using MeOH in the loading medium allowed use of the extract directly without evaporation, and it is known that a small percentage of organic solvent (MeOH) reduces non-specific interactions [30], PBS containing 15% MeOH was selected as the loading medium.

The kinetic study of antibody–antigen binding was performed by loading 40 mL of a 20 ng/mL CIP and SMX mixture in PBS/MeOH (85:15, v/v) onto the IAC at a flow rate of 0.5, 1, 1.5 and 2 mL/min. The columns were washed with 20 mL PBS followed by 30 mL of water and eluted with 4 mL of MeOH/water/ammonia (90:9.8:0.2, v/v/v) and detected by LC–MS/MS. An increase in the loading flow rate resulted in the decrease in recovery of both CIP and SMX. An increase in recovery from 61.4 to 95.3% and 56.9 to 91.2% for CIP and SMX, respectively, was observed when the flow rate decrease from 2 to 0.5 mL/min; however, the recoveries for CIP and SMX at flow rates of 0.5 and 1 mL/min were similar and for time-saving purposes, the flow rate at 1 mL/min was chosen for the subsequent study.

3.3.2. Washing conditions

Analytes of interest in matrix samples can be selectively captured by specific antibodies that are immobilized on a gel, and concomitantly the interfering matrix may also be retained because of non-specific binding to the gel, which may be caused by a sum of short-range ionic forces, π–π electron interactions, and hydrophobic interactions similar to that of antigen–antibody binding. These interfering components can often be removed by the wash procedure. In a previous report, the IAC was prepared using polyclonal antibodies and pure water by itself could not be used to eliminate the interfering matrix, but a small amount of organic solvent, MeOH, was required in the wash medium [7]. In our procedure, 40 mL of 20 ng/mL CIP and SMX in a solution of PBS/MeOH (85:15, v/v) was loaded onto the IAC at a flow rate of 1 mL/min, and then 20 mL of PBS followed by 30 mL of pure water was used to remove interfering components. The recovery of CIP and SMX was 92.3% and 94.5%, respectively, indicating that 20 mL of PBS combined with 30 mL of pure water used as wash medium was efficient to remove interfering components. The residual MeOH remaining following the loading procedure may play an important role in the washing procedure and the pure water was subsequently used to remove the PBS ions. If these ions were not removed they would cause severe ionization suppression of the analytes during the electrospray process.

3.3.3. Elution conditions

After the sample matrix is washed away, the trapped analyte can then be dissociated from the antibody–analyte complex with the elution solution. Previous workers have shown that mixtures of

Table 5
Influence of elution conditions on QN and SA recovery[a]

Elution medium	Recovery (%)	
	CIP	SMX
4 mL of 100% MeOH	54.7	93.2
4 mL of MeOH/water/acetic acid (90:9.8:0.2, v/v/v)	42.4	91.4
4 mL of MeOH/water/ammonia (90:9.8:0.2, v/v/v)	93.2	92.5
4 mL of 0.1 M ammonium acetate in MeOH	83.5	93.2
20 mL of 0.1 M glycin–HCl (pH, 3.0)	38.9	63.8

[a] Loading solution: 40 mL, 20 ng/mL of CIP and SMX in mixture of PBS containing 15% MeOH at flow rate of 1 mL/min. Washing solution: 20 mL of PBS followed by 30 mL water.

organic solvents (ACN or MeOH) were suitable to remove analytes from IAC [31–33]. We evaluated different elution buffers and solvents to find a better solvent solution that would obtain high QN and SA recoveries. After loading 40 mL (20 ng/mL) of CIP and SMX, the column was washed with 20 mL of PBS followed by 30 mL of water and then the CIP and SMX were eluted using five different elution solutions. The best recovery, 93.2% for CIP and 92.5% for SMX, was achieved by applying 4 mL of MeOH/water/ammonia (90:9.8:0.2, v/v/v) as the elution solution (Table 5). Elution with only MeOH resulted in recoveries of only 54.7% for CIP but up to 91.4% for SMX. High recoveries for CIP and SMX were also obtained using 4 mL of 0.1 M ammonium acetate in MeOH; however, the salt ions present in the elution buffer caused ionization suppression of the analytes during the electrospray process. It was concluded that up to 90% MeOH in the elution solution could remove SMX from the antibody in spite of the type of aqueous solution; however, a pure aqueous solution such as 0.1 M glycin–HCl (pH 3.0) was not suitable since it resulted in low recovery (<63.8%), even when the elution volume was increased to 20 mL. MeOH/water/ammonia (90:9.8:0.2, v/v/v) was the most satisfactory elution solution for CIP (Table 5). The

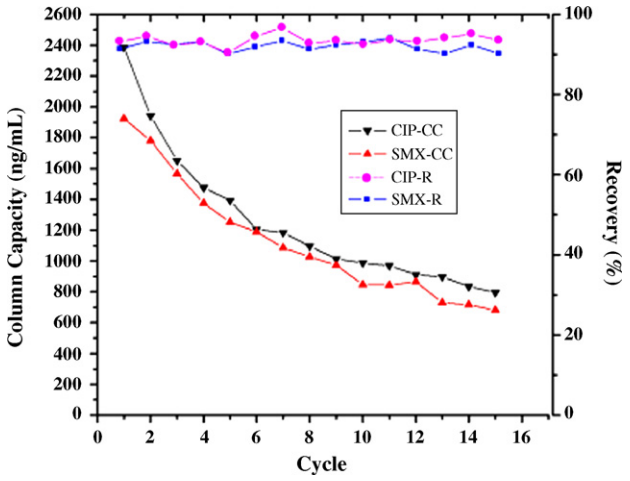

Fig. 1. The IAC capacity for CIP (CIP-CC) and SMX (SMX-CC) in ng/mL and variation in recovery of CIP (CIP-R) and SMX (SMX-R) in percent with 15 cycles of use over 45 days.

reason for this may be due to the ammonium, which can cause a change in pH and ionic strength. These changes may have a large influence on the short-range forces governing the binding in the antibody–analyte complex, resulting in release of the analyte from the gel.

3.4. Column capacity determination

After the loading, washing, flow rate, and elution conditions were optimized, the column capacity was determined by loading 6000 ng each of the 13 QN and 6 SA standard solutions at a flow rate

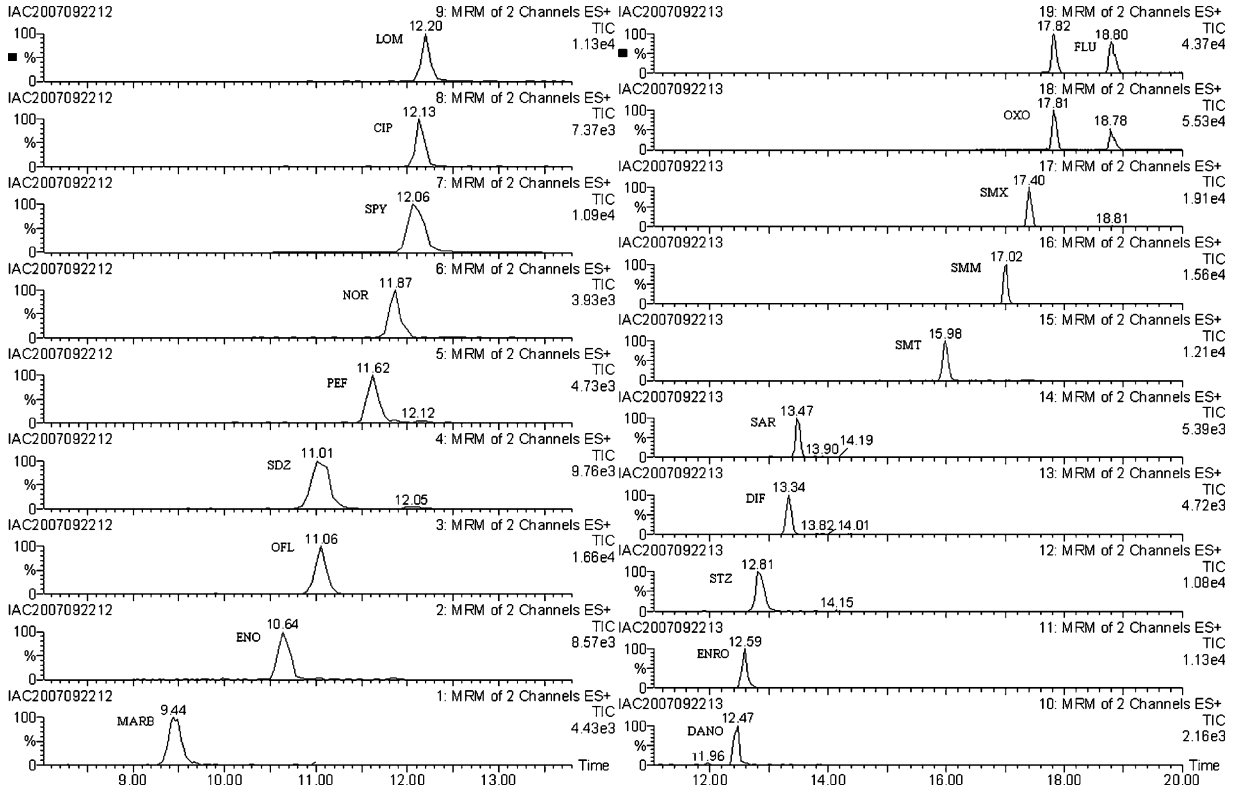

Fig. 2. Ion chromatograms of standard solutions of 2 ng/mL for OXO and FLU; 4 ng/mL for OFL, PEF, NOR, SPY, LOM, DANO, ENRO, STZ, DIF, SMM, and SMX; 8 ng/mL for ENO, SDZ, CIP, and SMT; 12 ng/mL for MARB and SAR. Standard solutions were obtained as follows: The mixed fortifying standard solutions was evaporated to dryness by stream of N_2 at 45 °C, and reconstituted in 0.5 mL of 0.1% formic acid solution.

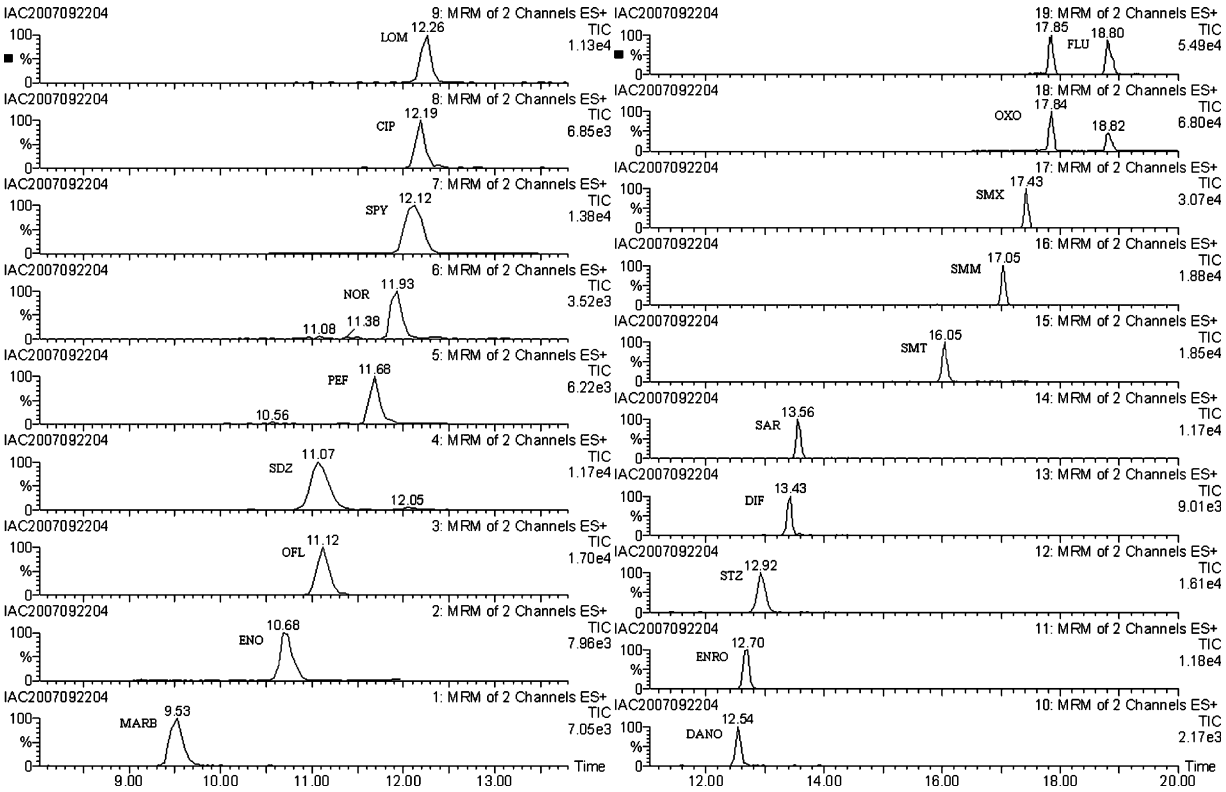

Fig. 3. Ion chromatograms of matrix-matched standard solutions of 2 ng/mL for OXO and FLU; 4 ng/mL for OFL, PEF, NOR, SPY, LOM, DANO, ENRO, STZ, DIF, SMM, and SMX; 8 ng/mL for ENO, SDZ, CIP, and SMT; 12 ng/mL for MARB and SAR. Matrix-matched standard solutions were obtained as follows: Control muscle tissue (2.0 g) was extracted and cleaned up as described in Section 2.9; The eluate was spiked with the mixed fortifying standard solutions; The spiked eluate was evaporated to dryness by stream of N_2 at 45 °C, and reconstituted in 0.5 mL of 0.1% formic acid solution.

of 1 mL/min. The IAC was washed with 20 mL PBS followed by 30 mL water, and eluted with 4 mL of MeOH/water/ammonia (90:9.8:0.2, v/v/v). The column capacity's for the QNs and the SAs are summarized in Table 3. The combination of the two Mabs have a high column capacity for all 19 drugs tested, exceeding 1 402 ng/mg for the QNs and 1834 ng/mg for the SAs, indicating that these columns are suitable for a class-selective extraction scheme. However, the column capacity for DIF and SAR should be noted because the lower affinity of the C4A9H1 Mab for DIF and SAR compared to that for other QNs seems to exert only a small influence on column capacity (1552 ng/mg for SAR and 1 402 ng/mg for DIF) despite the relatively lower antibody affinities. The reusability of the IAC was evaluated for CIP and SMX (used as model analytes) following 15 cycles of use in 45 days, and then stored in PBS for 2 days at 4 °C. The column capacity curve is shown in Fig. 1. The column capacity gradually decreases as the number of cycles increases; however, the percent recovery of CIP and SMX showed no loss over the 15 cycles of use (Fig. 1).

3.5. Method validation

The applicability of the developed method was tested following the accepted criteria for analytical method validation. Accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effects, and precision of the method were estimated based on the analyses of spiked animal muscle samples. Method accuracy was determined by calculating the mean of the percentage recoveries of the analyte concentrations in spiked animal muscle tissue. Method precision was expressed as relative standard deviation (RSD).

It is widely known that co-extracted matrix components can affect the ionization process in electrospray analyses. The matrix effects are related to both concentration and affinity for the protons (or cations) of the co-extracted and co-eluted matrix components [34]. To minimize this interference in the analysis of foodstuff matrices, some authors have proposed to use a matrix-matched calibration [34,35]. It was also demonstrated that matrix effects could be minimized or eliminated by adopting selective extraction methods [36]. Due to the high selectivity of our IAC, minimal matrix effects were observed upon comparing peak areas of standard solutions in 0.1% formic acid with the peak areas of the same analytes spiked before and after extraction of control muscle tissue (Figs. 2 and 3). Ion enhancement was observed for most of the analytes, especially for DIF and SAR. Minimal ion suppression was observed for ENRO, NOR, CIP and SMT in a number of cases.

However for reliable quantification, matrix-matched external standard calibration was employed in our analysis of muscle tissue samples to minimize potential matrix interferences. Control muscle tissue (2.0 g) was extracted and processed with cleanup procedures as described in Section 2.9. The eluate was spiked with mixed fortified standard solutions to obtain working standard solutions (Table 6). The spiked eluate was evaporated to dryness under a stream of N_2 at 45 °C, and reconstituted in 0.5 mL of 0.1% formic acid. After filtering through a 0.2 μm PTFE filter, the reconstituted samples were injected into the LC–MS/MS system. Linear calibration curves constructed by plotting the response factor for each analyte versus the matrix spiking concentrations were used to determine analyte concentrations in all subsequent analyses. The calibration curve for each analyte demonstrated excellent linearity with satisfactory correlation coefficients (r) ranging between 0.9909 and 0.9992 (Table 6).

The LOD and LOQ of the method were determined by measuring the peak height of the analytes compared to control chicken and swine muscle samples ($n = 10$), and were based on a signal-to-noise ratio of 3:1 and 10:1. The LOQs were 0.5 ng/g for OXO and FLU,

Table 6
Matrix-matched standard curve ($n = 6$) parameters; LOD and LOQ of the method

Analytes	Linear range (ng/mL)	Working standard solutions (ng/mL)	Slope	Intercept	r	LOD (ng/g)	LOQ (ng/g)
MARB	3–300	3, 12, 24, 60, 120, 300	66.77	−395.51	0.9924	0.9	3.0
ENO	2–200	2, 8, 16, 40, 80, 200	95.91	−79.13	0.9989	0.6	2.0
OFL	1–100	1, 4, 8, 20, 40, 100	489.18	−458.08	0.9983	0.3	1.0
SDZ	2–200	2, 8, 16, 40, 80, 200	208.83	420.99	0.9955	0.6	2.0
PEF	1–100	1, 4, 8, 20, 40, 100	142.01	231.77	0.9931	0.3	1.0
NOR	1–100	1, 4, 8, 20, 40, 100	205.10	0.22	0.9977	0.3	1.0
SPY	1–100	1, 4, 8, 20, 40, 100	443.20	389.53	0.9968	0.3	1.0
CIP	2–200	2, 8, 16, 40, 80, 200	148.67	−725.43	0.9912	0.6	2.0
LOM	1–100	1, 4, 8, 20, 40, 100	370.36	−888.53	0.9946	0.3	1.0
DANO	1–100	1, 4, 8, 20, 40, 100	282.35	−389.29	0.9971	0.3	1.0
ENRO	1–100	1, 4, 8, 20, 40, 100	402.51	−348.06	0.9991	0.3	1.0
STZ	1–100	1, 4, 8, 20, 40, 100	489.38	−426.63	0.9990	0.3	1.0
DIF	1–100	1, 4, 8, 20, 40, 100	266.77	−552.18	0.9921	0.3	1.0
SAR	3–300	3, 12, 24, 60, 120, 300	103.35	−551.50	0.9946	0.9	3.0
SMT	2–200	2, 8, 16, 40, 80, 200	184.51	−963.28	0.9932	0.6	2.0
SMM	1–100	1, 4, 8, 20, 40, 100	299.68	−318.61	0.9986	0.3	1.0
SMX	1–100	1, 4, 8, 20, 40, 100	480.32	−488.52	0.9992	0.3	1.0
OXO	0.5–50	0.5, 2, 4, 10, 20, 50	1332.20	1584.10	0.9909	0.15	0.5
FLU	0.5–50	0.5, 2, 4, 10, 20, 50	1815.10	−1149.80	0.9976	0.15	0.5

Table 7
Recoveries and RSD for 13 QNs and 6 SAs in swine and chicken muscle

Analytes	Spikes (ng/g)	Sample	Intra-day ($n = 6$)		Inter-day ($n = 3$)	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
MARB	3, 12, 30, 90	Swine	76.5–104.1	4.0–9.4	77.9–103.3	5.6–15.4
		Chicken	78.9–96.8	5.1–8.6	76.6–101.0	6.2–10.6
ENO	2, 8, 20, 60	Swine	77.2–86.6	4.5–10.3	74.5–93.4	3.4–11.0
		Chicken	76.0–87.4	7.3–8.0	80.6–98.2	6.3–10.8
OFL	1, 4, 10, 30	Swine	75.9–91.1	4.9–6.5	79.4–96.5	4.4–9.6
		Chicken	75.2–89.9	5.6–9.8	78.8–94.0	4.7–10.9
SDZ	2, 8, 20, 60	Swine	74.5–94.4	6.5–9.0	81.5–97.7	5.6–10.3
		Chicken	83.7–96.5	5.2–8.7	82.4–95.4	4.6–7.4
PEF	1, 4, 10, 30	Swine	84.0–91.8	3.6–9.1	85.9–96.0	4.2–11.1
		Chicken	77.7–94.3	4.3–8.3	76.0–98.9	5.6–9.0
NOR	1, 4, 10, 30	Swine	80.1–97.4	6.7–8.0	81.2–90.6	7.1–10.2
		Chicken	78.3–90.5	5.9–7.4	77.0–91.1	6.5–8.7
SPY	1, 4, 10, 30	Swine	76.4–89.3	6.3–8.9	74.8–92.5	6.3–8.9
		Chicken	77.2–95.5	6.9–8.5	85.7–92.2	5.8–9.7
CIP	2, 8, 20, 60	Swine	81.7–99.4	3.5–7.4	85.0–107.6	4.3–9.6
		Chicken	83.0–95.6	5.0–8.8	83.9–104.4	6.5–10.6
LOM	1, 4, 10, 30	Swine	79.0–97.6	3.8–7.7	76.7–98.0	4.7–12.0
		Chicken	84.2–96.5	4.4–9.0	79.1–94.3	6.3–9.8
DANO	1, 4, 10, 30	Swine	74.7–93.8	5.6–11.1	79.9–92.5	4.5–13.4
		Chicken	78.5–88.1	6.8–9.4	78.5–97.8	5.6–10.9
ENRO	1, 4, 10, 30	Swine	82.1–96.2	4.1–8.2	89.3–99.9	5.3–9.7
		Chicken	80.0–97.8	5.3–7.6	78.5–94.3	6.2–10.6
STZ	1, 4, 10, 30	Swine	76.5–88.6	3.6–8.0	73.5–90.1	4.5–7.7
		Chicken	78.4–91.1	4.5–6.9	76.0–88.9	5.3–9.9
DIF	1, 4, 10, 30	Swine	82.7–98.0	6.5–11.3	80.4–94.4	4.6–13.6
		Chicken	75.4–90.3	5.0–9.7	76.2–91.2	6.1–11.7
SAR	3, 12, 30, 90	Swine	78.3–87.9	6.8–10.1	85.1–93.8	5.3–9.4
		Chicken	75.2–86.6	5.4–9.3	78.4–92.3	6.4–12.1
SMT	2, 8, 20, 60	Swine	84.7–93.4	5.9–9.9	81.9–97.5	4.8–7.4
		Chicken	80.6–96.0	4.7–9.5	85.8–103.5	3.7–11.4
SMM	1, 4, 10, 30	Swine	77.6–89.4	3.5–7.2	75.4–93.2	3.4–8.7
		Chicken	78.8–90.0	4.0–8.8	78.0–93.2	4.9–10.5
SMX	1, 4, 10, 30	Swine	72.6–88.5	3.9–7.6	74.3–91.6	4.3–9.7
		Chicken	76.8–92.4	4.3–9.5	76.9–93.0	3.3–10.8
OXO	0.5, 2, 5, 15	Swine	85.9–95.2	3.1–6.7	81.1–98.3	5.0–8.7
		Chicken	81.6–94.0	2.6–8.3	78.4–89.0	4.6–9.4
FLU	0.5, 2, 5, 15	Swine	81.2–95.3	4.1–6.4	76.8–92.8	6.0–12.3
		Chicken	83.0–91.1	4.4–9.0	81.8–94.3	5.7–7.8

1 ng/g for OFL, PEF, NOR, SPY, LOM, DANO, ENRO, STZ, DIF, SMM and SMX, 2 ng/g for ENO, SDZ, CIP and SMT, 3 ng/g for MARB and SAR, respectively. The accuracy and precision of six replicate blank tissue samples fortified at the LOQ level for each analyte was determined. The average recoveries were between 60 and 110% with RSD less than 20%. The accuracy and precision at this level fall within the criteria set by the FDA [37]. Due to the high cleanup performance of the IAC and the specificity of tandem MS, only minor background peaks were observed in control chicken and swine muscle samples. LOQs of the method are well below the MRLs set for QNs and SAs recommended by the EU and the United States [2,3].

The accuracy and precision of the method was evaluated at four different concentrations using spiked muscle tissue samples fortified with the mixed standard solutions. At each concentration, six measurements from intra-day experiments and three measurements from inter-day experiments were performed. The recovery results were summarized in Table 7. The mean recovery for each analyte in spiked samples ranged from 72.6% to 104.1% with the RSD between 2.6 and 11.3% during intra-day experiments, and ranged from 73.5% to 107.6% with the RSD between 3.3 and 15.4% during inter-day experiments. The results demonstrated that MeOH/water (8:2, v/v) (Section 2.9) was the solvent of choice for simultaneously extracting both QNs and SAs from chicken and swine muscle.

4. Conclusions

In this study, an IAC/LC–MS/MS method was successfully developed that takes advantage of the positive feature of high cross-reactivity of two broad-specificity Mabs for simultaneously trapping two groups of analytes, QNs (13 analogs) and SAs (6 analogs). These antibiotics are the most widely used in veterinary medicine and were chosen as target models. After optimizing the IAC operational parameters, they were used to selectively isolate QNs and SAs from swine and chicken muscle samples. Compared to conventional IAC, which uses only a single antibody, mixed-bed IAC demonstrated its ability to co-extract and co-cleanup two groups of analytes, and was less time-consuming and less costly. The satisfactory recovery obtained with the mixed-bed IAC suggests that it could be a promising one step sample pretreatment tool for extraction and cleanup of several groups of analytes present in complicated matrices. The strategy described in this paper for QNs and SAs represents a generic approach that may be applied to the determination of other veterinary drugs in food samples of animal-origin.

Acknowledgments

This work was supported by the Chinese National Outstanding Youth Science Fund (30325032) and National Key Technology & Program (2006BAK02A08).

References

[1] C. Blasco, C.M. Torres, Y. Picó, Trends Anal. Chem. 26 (2007) 895.
[2] J.A. Hernández-Arteseros, J. Barbosa, R. Compañá, M.D. Prat, J. Chromatogr. A 945 (2002) 1.
[3] M. Franek, I. Diblikova, I. Cernoch, M. Vass, K. Hruska, Anal. Chem. 78 (2006) 1559.
[4] R.S.M. Cichna-Markl, J. Sol-Gel Sci. Technol. 41 (2007) 175.
[5] J.C. Chuang, J.M. Van Emon, R. Jones, J. Durnford, R.A. Lordo, Anal. Chim. Acta 583 (2007) 32.
[6] K. Schöringhumer, M. Cichna-Markl, J. Chromatogr. B 850 (2007) 361.
[7] J.S. Li, X.W. Li, J.X. Yuan, X. Wang, J. AOAC Int. 83 (2000) 830.
[8] A. Martin-Esteban, P. Kwasowski, D. Stevenson, Chromatographia 45 (1997) 364.
[9] V. Pichon, M. Bouzige, M.-C. Hennion, Anal. Chim. Acta 376 (1998) 21.
[10] D. Chan, S.J. MacDonald, V. Boughtflower, P. Brereton, J. Chromatogr. A 1059 (2004) 13.
[11] M.W. Trucksess, C.M. Weaver, C.J. Oles, L.V. Rump, K.D. White, J.M. Betz, J.I. Rader, J. AOAC Int. 90 (2007) 1042.
[12] P. Crabbe, C. Van Peteghem, W. Haasnoot, F. Kohen, M. Salden, Analyst 124 (1999) 1569.
[13] B. Toussaint, M. Chedin, U. Vincent, G. Bordin, A.R. Rodriguez, J. Chromatogr. A 1088 (2005) 40.
[14] Y. Wen, M. Zhang, Q. Zhao, Y.-Q.J. Feng, Agric. Food Chem. 53 (2005) 8468.
[15] E. Märtlbauer, R. Dietrich, E. Usleber, ACS Symp. Ser. 636 (1996) 121.
[16] W. Heering, E. Usleber, R. Dietrich, E. Märtlbauer, Analyst 123 (1998) 2759.
[17] C.K. Holtzapple, S.A. Buckley, L.H.J. Stanker, Agric. Food Chem. 45 (1997) 1984.
[18] H. Zhang, Z. Duan, L. Wang, Y. Zhang, S.J. Wang, Agric. Food Chem. 54 (2006) 4499.
[19] Z. Wang, Y. Zhu, S. Ding, F. He, R.C. Beier, J. Li, H. Jiang, C. Feng, Y. Wan, S. Zhang, Z. Kai, X. Yang, J. Shen, Anal. Chem. 79 (2007) 4471.
[20] A.-C. Huet, C. Charlier, S.A. Tittlemier, G. Singh, S. Benrejeb, P.J. Delahaut, Agric. Food Chem. 54 (2006) 2822.
[21] S. Zhang, J. Zhou, J. Shen, S. Ding, J. Li, J. AOAC Int. 89 (2006) 369.
[22] E. Märtlbauer, R. Meier, E. Usleber, G. Terplan, Food Agric. Immunol. 4 (1992) 219.
[23] S. Zhang, Z. Wang, I.S. Nesterenko, S.A. Eremin, J. Shen, Int. J. Food Sci. Technol. 42 (2007) 36.
[24] J. He, X. Hou, H. Jiang, J. Shen, J. AOAC Int. 88 (2005) 1099.
[25] M.-C. Hennion, V. Pichon, J. Chromatogr. A 1000 (2003) 29.
[26] W. Zhang, H. Wang, J. Wang, X. Li, H. Jiang, J. Shen, J. AOAC Int. 89 (2006) 1677.
[27] J. Dallüge, T. Hankemeier, R.J.J. Vreuls, U.A.Th. Brinkman, J. Chromatogr. A 830 (1999) 377.
[28] C.K. Holtzapple, E.J. Pishko, L.H. Stanker, Anal. Chem. 72 (2000) 4148.
[29] S. Ding, Y. Hou, N. Wu, J. Shen, J. AOAC Int. 88 (2005) 1644.
[30] N. Delaunay, V. Pichon, M.-C. Hennion, J. Chromatogr. B 745 (2000) 15.
[31] Z. Brenn-Struckhofova, M. Cichna-Markl, C. Böhm, E. Razzazi-Fazeli, Anal. Chem. 79 (2007) 710.
[32] P.L. Ferguson, C.R. Iden, A.E. McElroy, B.J. Brownawell, Anal. Chem. 73 (2001) 3890.
[33] S. Ouyang, Y. Xu, Y.H. Chen, Anal. Chem. 70 (1998) 931.
[34] U. Koesukwiwat, S. Jayanta, N. Leepipatpiboon, J. Chromatogr. A 1140 (2007) 147.
[35] S. Yang, J. Cha, K. Carlson, J. Chromatogr. A 1097 (2005) 40.
[36] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.
[37] U.S. Food and Drug Administration, Center for Veterinary Medicine, Guideline for approval of a method of analysis for residues. http://www.fda.gov/cvm/guidance/1731.htm.